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(71) Applicant: GEL TECH GROUP INC. [US/US]; 545 West 37th Street, Miami, FL 33140 (US).

(72) Inventors: HICKMAN, Suzanne; Apartment 5F, 499 Fort Washington Avenue, New York, NY 10033 (US). LOIKE, John, D.; 179-20 Tudor Road, Jamaica, NY 11432 (US). HOLZER, David; 545 West 37th Street, Miami Beach, FL 33140 (US).

(74) Agents: JACOBS, James, David et al.; Rosen, Dainow & Jacobs, Limited Liability Partnership, 489 Fifth Avenue, New York, NY 10017 (US).

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(57) Abstract

Described is a method of producing a collagen compound in transgenic plants. The compound is preferably procollagen or collagen or fragments thereof of human, animal or fish origin. The compound may be recovered from the plant as collagen or gelatin.

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COLLAGEN COMPOUND PRODUCTION IN PLANTS

FIELD OF THE INVENTION

This invention relates to plant genetic engineering and more particularly to the expression of foreign proteins in plants and specifically to recombinant gene constructs used to introduce foreign proteins into plants and to transgenic plants and their seeds which express a human, animal or fish protein.

BACKGROUND OF THE INVENTION

Collagen, in the form of insoluble fibers or fibrous aggregates, is the major proteinaceous constituent of most vertebrates and many invertebrates. In mammals, collagen is the most abundant body protein forming the major protein constituent of connective tissue, skin, tendon. cartilage and bone.

One type of collagen, collagen I, is the major component of gelatin, a protein useful in food production as a jellying agent, stabilizer, emulsifier, thickener, foaming agent, water binder, crystal growth modifier, glaze, adhesive, binder and fining agent. When blended into milk, gelatin adds a consistency and desirable "mouth feel" to the milk. Gelatin used in the food industry is typically obtained by isolating collagenous material from animal skins and bones or from fish by means of an extensive extraction process. This typically requires several days of treating the hides and bones with lime and/or acids and includes several washing steps.

There is a growing demand for gelatin suitable for the needs of the kosher, Muslim and Seventh Day Adventist consumer. These consumers have dietary restrictions against consumption of pork and pork products. Most commercially available gelatin is produced from pork. In the past, only small quantities of properly certified kosher or non-pork gelatin has been produced from limited supplies of hides and bones derived from kosher animals or from skins of kosher fish. The price of kosher gelatin ranges from 6 to 15 times the price of standard bovine or porcine gelatin. The preparation of kosher gelatin is expensive and the limitations of raw material make its production in large quantities on a regular basis extremely difficult.

There is, therefore, a need for a method of producing a ready supply of kosher gelatin which is inexpensive to prepare.

Gelatin is one of the most versatile natural products known. It is a material with a wide range of physical and biochemical properties which are responsible for the numerous and varied applications in which gelatin plays an important part. Gelatin is useful in the photographic and leather industries and for pharmaceutical and medical uses, printing applications, coated abrasives and adhesive uses, among other things. Possibly the most important single property of gelatin is its gel-forming characteristic. Many of the other physical properties of gelatin, such as its protective-colloidal, setting, swelling and film-forming properties are useful in the photographic process, paper-making and textile industry.

There is therefore a need for large quantities of gelatin which have the biochemical and biophysical properties required for use in the photographic, pharmaceutical/medical, leather, glue, printing and other industries.

Industrial uses of corn are mainly from corn starch in the wet-milling industry and corn flour in the dry-milling industry. The industrial uses for corn starch and corn flour are based on functional properties, such as viscosity, film formation, adhesive properties, and ability to suspend particles. Corn starch is an example of a class of food gums known as seed gum. Besides the usefulness of corn starch as a food thickener, both corn starch and corn flour have applications in the paper and textile industries, as well as in connection with adhesives, building materials, foundry binders, laundry starches, explosives, oil-well muds, and other mining applications.

Collagen is useful in medical, reconstructive, therapeutic and cosmetic applications in humans. For instance, collagen injection therapy is used to treat urinary incontinence (Appell, R.A., Urologic Clinics of North American 21:177-182 (1994)); photodamaged skin (Goldhar et al., Canadian Family Physician 39:352-356, 359-363 (1993)); acne scars (Matsuoka, L.Y., Clinics in Plastic Surgery 20:35-41 (1993); Low et al., Journal of Dermatologic Surgery & Oncology 18:981-986 (1992)); peridontal tissue regeneration (Dowell et al., British Dental Journal 171:125-127 (1991)) and as a drug delivery system to target drugs for the eye (Friedberg et al., Ophthalmology 98:725-732 (1991)). Collagen for these purposes is typically obtained from tissues of farm animals such as cows or pigs. As such collagen is essentially a foreign protein, immunogenic responses may present problems. To some extent, these problems may be minimized by treating the animal-derived collagen to decrease immunogenicity. However, there remains a need for a ready supply of human collagen for pharmacological, therapeutic and cosmetic applications.

In our co-pending application, Serial No. 08/445,254 filed June 2, 1995, is described a method for producing in milk of a female, non-human mammal a compound endogenous to the mammal but not naturally secreted in its milk and of generating an isogenic mammal which has been genetically altered to produce in its milk an endogenous compound which is normally secreted by other tissues in the mammal. The compound is preferably procollagen or collagen or fragments thereof, which may easily be converted to gelatin. The compound may be recovered from the collected milk or used directly in food production. The disadvantage is that the gelatin obtained from the collagen produced in the milk is dairy, which, for the kosher consumer, limits its use to dairy products only. There is a need for a non-mammalian and non-dairy source of gelatin for the vegetarian market. Furthermore, a special extraction process which is not typically part of milk processing is required to recover collagen or gelatin from the milk of isogenic mammals. Additionally, significant time is required to produce a herd of isogenic milk producing mammals.

Plant genetic engineering techniques are utilized to obtain plants having improved characteristics or traits and to incorporate heterologous genes from a source other than the transformed plant into plant cells to obtain desired traits or to produce useful polypeptides in the plant cells. Thus, transgenic plants have been developed to produce crops of increased value.

Transgenic plants have been developed which produce foreign biological proteins. The 5' regulatory region and putative signal sequence of a rice alpha amylase gene was fused to a bacterial gene encoding β-glucuronidase (GUS) and introduced into rice, tobacco, and potato via Agrobacterium-mediated transformation systems. Expression of alpha amylase was suppressed by the presence of sucrose in the medium and induced by its absence. Ming-Tsair Chan et al., J. Biol. Chem. 269:17635-17641 (1994). Turpen et al., Bio/Technology 13:53-57 (1995), disclose malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. The authors engineered hybrid virions of tobacco mosaic virus that contain malaria-parasite immunodominant sequences recognized by protective monoclonal antibodies against these parasites and that yet retain their infectivity.

Herbers et al. in Bio/Technology 13:63-66 (1995) disclose expression at high levels of a thermostable, microbial xylanase from *Clostridium thermocellum* in the apoplast of transgenic tobacco. The expressed xylanase was easily purified.

Tobacco plants were genetically transformed with the gene encoding hepatitis B surface antigen (HBsAg) linked to a nominally constitutive promoter. The transgenic plants produced HBsAg which is antigenically and physically similar to the HBsAg particles derived from human serum and recombinant yeast. Mason et al. in Proc. Nat. Acad. Sci., USA, 89:11745-11749 (1992). The immunological response elicited *in vivo* by using recombinant HBsAg purified from transgenic tobacco leaves was qualitatively similar to that obtained by immunizing mice with yeast-derived recombinant HBsAg (commercial vaccine). Both B- and T-cell epitopes are preserved when the antigen is expressed in transgenic tobacco. Thanavala et al. in Proc. Nat. Acad. Sci. USA, 92:3358-3361 (1995).

Mori et al. in FEBS 13403, vol. 336, no. 1, 171-174 (1993), disclose construction of transgenic tobacco plants expressing viral RNA replication genes of brome mosaic virus (BMV) and BMV RNA3 derivative carrying the human gamma interferon gene.

The genes encoding the heavy and light chains of a mouse monoclonal antibody (mAb Guy's 13) have been cloned and expressed in tobacco plants, *Nicotiana tabacum*. Transgenic plants were regenerated that secrete full-length Guy's 13 antibody. Antigen binding studies confirmed the fidelity of assembly and demonstrated that the antibody is fully functional. Furthermore, the plant antibodies retained the ability to aggregate streptococci, which confirms that the bivalent antigen-binding capacity of the full length antibodies is intact. The results demonstrate that IgA as well as IgG class antibodies may be assembled correctly in tobacco plants and suggest that transgenic plants may be suitable for high-level expression of more complex genetically engineered immunoglobulin molecules. Ma, et al. in Eur. J. Immunol. 24:131-138 (1994).

Plant genetic engineering is employed to obtain plants having improved characteristics or traits, such as virus resistance, insect resistance, herbicide resistance, enhanced stability, improved plant taste or nutritional value, altering, starch, oil and protein profile, yield or quality. For instance, work is being done on tomatoes that may be vine-ripened and shipped without bruising, and to provide tomatoes which are better tasting, have improved color and higher vitamin content and which contain more solids. Other projects are directed to generate tomatoes with improved viscosity, i.e., thickness and texture, which means fewer tomatoes are required to generate the same amount of catsup.

Pectin, used to make jelly thicken or gel, occurs naturally in many fruits and vegetables, giving them their firmness. The pectin in ripening tomatoes is degraded by the

enzyme polygalacturonase (PG). As pectin is destroyed, the cell walls of tomatoes break down and soften, making them difficult to successfully ship to market. Reducing the amount of PG in tomatoes slows cell wall breakdown and results in a fruit which remains firm for a longer time at ambient temperature. Calgene, Inc. of Davis, California developed a tomato which incorporates a gene that essentially attaches itself to the PG gene and inactivates it. Such transgenic plants produce drastically reduced levels of PG. This slows the natural softening process that accompanies ripening and allows the Flavr Savr™ tomato to spend more time on the vine than other tomatoes. This results in a more flavorful tomato which is firm enough to be shipped.

U.S. Patent No. 5,202,422 to Hiatt et al. issued April 13, 1993 discloses a method for producing a glycopolypeptide multimer by introducing first and second mammalian genes encoding the constituent parts of the multimer into first and second respective members of a plant species, generating a progeny from the first and second plant species members, and isolating the glycopolypeptide multimer from the progeny plant.

U.S. Patent No. 5,188,958 to Moloney et al. issued February 23, 1993 discloses transgenic Brassica species cells (such as rapeseed and rutabaga) produced by transformation of cell cultures with foreign DNA which when expressed will alter the phenotype of the transgenic cells using a manipulated Agrobacterium transformation system followed by regeneration of plants from transformed cells. An example of such foreign DNA is the gene for kanamycin resistance. The cells and the plants produced thereby are capable of expressing the foreign gene.

U.S. Patent No. 5,384,253 to Krzyzek et al. issued January 24, 1995 discloses a method to increase the susceptibility of cultured *Zea mays* cells to stable transformation with recombinant DNA via electroporation so that the cells retain their ability to regenerate fertile, transgenic *Zea mays* plants containing the introduced DNA which may be inherited by progeny of the transformed plant.

D'Halluin et al., Transgenic Maize Plants by Tissue Electroporation, The Plant Cell, 4:1495-1505 (1992) describe the production of normal, fertile transgenic monocots by creating transformation-competent cells from immature zygotes and type I callus by gentle partial hydrolysis of certain cell wall components, such as pectin. This results in enhanced permeability of the cell wall to exogenous DNA while not destroying cell viability.

One problem with using transgenic plants to express foreign genes is that plants may not always properly fold or process mammalian proteins and may lack post-translational enzymes that are mammalian specific so as to provide the desired degree of biological or physiological activity in the resulting protein.

OBJECTS AND SUMMARY OF THE INVENTION

It is an object of the invention to provide a method of producing a ready supply of gelatin from a specific non-mammalian source.

It is an object of the invention to provide a method of producing a collagen compound in plants.

It is another object of the invention to provide a method of inexpensively producing high yields of kosher gelatin in a short period of time.

It is a further object of the invention to provide a method of producing an inexpensive. ready supply of human collagen and procollagen for medical and cosmetic applications.

Another object of the invention is to provide a method of producing a ready supply of gelatin by producing a collagen compound in plants by genetic manipulation.

It is another object of the invention to provide a method of producing large quantities of gelatin having the biochemical and biophysical properties suitable for use in the food, photographic, pharmaceutical/medical, paper, leather and glue industries.

It is yet another object of the invention to provide a method of producing large quantities of non-Bloom gelatin suitable for microencapsulation and photographic applications.

It is also an object of the invention to provide a method of producing gelatin from plants from which food gums are extracted to achieve an enhanced gelatin effect.

A still further object of the invention to provide a gene construct for producing a collagen compound in plants.

Another object of this invention is the development of transgenic plants capable of reproduction which produce a collagen compound.

Still another object of the present invention is the production of a low cost collagen compound in plants.

Yet another object of the invention is to provide a method of easily and inexpensively producing human collagen for therapeutic and cosmetic uses.

An additional object of the invention is the development of transgenic corn which produces a collagen compound.

These and other objects of the invention are accomplished by providing a method of producing transgenic plants capable of expressing a collagen compound. The method comprises introducing a gene construct into cells of a plant. The gene construct comprises a DNA sequence coding for the collagen compound operably linked upstream (5') to a plant promoter sequence capable of initiating and directing transcription in plant cells of the DNA sequence. The gene construct is integrated into the plant genome such that, upon transformation, the plant cells express the collagen compound. Transgenic plants are regenerated from the transformed plant cells and grown for a time and under conditions sufficient to permit expression of the collagen compound. The collagen compound is then recovered from the transgenic plants.

The collagen compound is preferably procollagen, collagen I through collagen XIX or fragments thereof. The collagen may be of human, animal or fish origin, and preferably of bovine origin. Specifically, the collagen compound may comprise collagen I alpha 1, collagen I alpha 2 and other recombinant collagen products, such as collagen II through collagen XIX, inclusive, and fragments thereof. The procollagen forms of bovine collagen I through XIX and fragments thereof may also be produced by the methods of this invention.

Additionally, in an embodiment of the invention, a gene construct comprises at least a DNA sequence coding for the collagen compound operably linked upstream (5') to a promoter sequence capable of initiating and directing transcription in plant cells of the DNA sequence. When such a gene construct is incorporated into a plant genome. the DNA sequence coding for the collagen compound is expressed in the plant's cells.

In further embodiments of the invention, there is provided a collagen compound-producing plant cell produced by the method described above and a collagen-compound producing plant cell descended from the above plant cell. In yet another embodiment of the invention, there is provided collagen produced from plants and seeds, gelatin produced from plants and seeds and a dairy or vegetarian food product which includes gelatin produced from plants.

In other aspects, the invention is directed to plant cells transformed with the gene constructs described above and to plants regenerated from or containing these transformed plant cells. In further aspects, the invention is directed to methods to produce plants and

seeds which produce a collagen compound which method comprises cultivation of the transgenic plants of the invention followed by recovery of the collagen compound as collagen or gelatin.

The invention contemplates genetically altering plants, preferably corn plants, to program them to synthesize any one or more of the collagen proteins I through XIX, procollagen or any collagen fragments of human, animal or fish origin. The collagen producing corn plants are used as an inexpensive method to generate plant derived gelatin. In addition, gelatin from such plants is valuable in the manufacturing of gelatin coated capsules for prescription and over-the-counter pharmaceuticals. Corn is the preferred plant as corn starch is an essential filler substance used in the pharmaceutical industry in making pills and capsules. Thus, the transgenic corn plants of the invention are a source of both corn starch and gelatin, the major ingredients for gelatin coated capsules. The vegetarian collagen compound of the invention has utility in food production and in pharmacology, medicine, agriculture and the cosmetics industry. An additional advantage of using corn as the transgenic plant is the recovery of collagen or gelatin without added cost as technology is already well developed to extract protein from corn, for instance, by a wet milling process. For non-pharmaceutical uses, the recovered collagen or gelatin is not required to be of high purity.

The collagen compounds that may be produced by the methods of this invention include, for instance, collagen I and fragments thereof and specifically, collagen I alpha 1 and collagen I alpha 2 and other recombinant collagen products, such as collagen II through collagen XIX, inclusive, and fragments thereof. The procollagen forms of collagen I through XIX and fragments thereof may also be produced by the methods of this invention. The collagen compound may be of human, animal or fish origin. In its broadest applications, the present invention is not limited to any particular species of mammals or fish. For instance, mammals such as swine, goat, sheep, oxen and cattle may be used and cold and warm water fish may be used. Advantageously, kosher mammals and fish are used. The preferred fish are carp (*Cyprinus Carpio*), tilapia (*Tilapia Nilotica* and the like) and Nile Perch (*Lates Niloticus*). In these fish, gelatin having a Bloom strength in the same range as hide gelatins (about 250-300 Bloom) may be derived but their melting points are in the range of approximately three degrees to six degrees centigrade lower than in bone gelatin. Standard

Knox ® 300 Bloom gelatin (derived from bovine bone) has a melting point of 29°C, while fish gelatins have a melting point in the range of
23 - 26°C.

It is contemplated that the method of the invention may be used to tailor-make collagen or gelatin that meets the needs of a specific industry or application. For instance, where gelatin with a high Bloom strength and low melting point is required for a certain application, it is possible to prepare a transgenic plant which expresses collagen from which such a gelatin having the desired characteristics may be recovered by providing an appropriate gene construct comprising a DNA sequence coding for a suitable collagen compound.

These and other features, objects and advantages of the invention will be apparent from the following description of the preferred embodiment, taken in conjunction with the accompanying drawings and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is illustrated by way of example and not limitation in the Figures of the accompanying drawings in which like references denote like or corresponding parts, and in which:

Fig. 1 shows a gene construct according to the invention; and

Fig. 2 shows an alternative gene construct according to the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides a method of producing transgenic plants capable of expressing a collagen compound comprising introducing a gene construct into cells of a plant. The gene construct comprises a DNA sequence coding for the collagen compound operably linked upstream (5') to a promoter sequence capable of initiating and directing transcription in plant cells of the DNA sequence. The gene construct is integrated into the plant genome such that upon transformation the plant cells express the collagen compound. Transgenic plants are regenerated from the plant cells which have been transformed and grown for a time and under conditions sufficient to permit expression of the collagen compound.

The present invention is capable of providing an unlimited supply of kosher source gelatin. with the long term costs being less than that of standard gelatin. By providing transgenic collagen-compound producing plants, there will never be a shortage of raw material. The present invention contemplates producing transgenic corn cells via

electroporation which are capable of regeneration to yield fertile, transgenic corn plants. The transgenic corn plants produce a collagen compound, specifically procollagen or any member of the collagen family I through II or fragments thereof. Ordinarily, corn does not produce collagen in any form.

As a result of integration of the gene construct in the plant genome, the gene construct will be present in all or substantially all of the cells of the plant tissue after transformation and regeneration. Alternatively, expression of the collagen compound gene may be targeted to particular plant tissue or particular stages in the development of the plant, by the use of tissue or organ specific, or developmental stage specific promoters which can be expressed in the plant cell of choice.

Since collagen I is the major component of gelatin, gene constructs are introduced into a plant, preferably corn, which instructs the plant to produce a collagen compound in its cells. The collagen compound is extracted from corn as collagen or gelatin using known wet milling technology. As the destabilization temperature of collagen I is usually above 41° C., a 2-4 minute heat treatment of the collagen compound at or above this temperature and subsequent cooling to room temperature or below, i.e., approximately 25° C or below, will convert the collagen compound to gelatin. Alternatively, heat treatment for a longer period at lower temperatures and subsequent cooling to room temperature or below will convert the collagen compound to gelatin. While procollagen or collagen does not automatically have any gelling characteristics, measured by Bloom strength, the method of the invention contemplates obtaining gelatin preferably with a Bloom strength in the range of 0 to 350 Bloom and preferably in the range of 90-300 Bloom, i.e., from non-Bloom gelatin to full-strength Bloom gelatin, and possessing the biochemical and biophysical properties necessary to meet the particular applications and needs of the food production, medical, therapeutic, cosmetic, photographic, glue, leather and other industries.

There is also a need for non-Bloom gelatin for applications such as microencapsulation and photography. Microencapsulation involves safely enclosing materials in microcapsules for later use for such purposes as holding an agent for controlled initiation of a chemical reaction, for controlling the release rate or masking the taste of pharmaceuticals, or for timely release of a flavor or odor. Non-Bloom gelatin is typically obtained from fish collagen and is stable, i.e., it does not gel, at room temperature. The preferred fish collagen for this purpose is obtained from cod, haddock and pollock, but may

be obtained from other fish as well. Accordingly, the method of the invention contemplates obtaining non-Bloom gelatin for use in microencapsulation and photography applications. For applications calling for a higher Bloom gelatin having a lower melting point than standard gelatin, it is contemplated that a DNA sequence coding for a collagen compound obtained from tilapia fish (*Tilapia Nilotica* and the like), carp (*Cyprinus Carpio* of the Cyprinidae family) and Nile Perch (*Lates Niloticus),* among others, is suitable. Such gelatin is useful in producing soft gelatin capsules.

Definitions

The terms plant, selectable marker genes and reporter genes, transcription termination sequences, transformation and operably linked have the following meanings in this application and claims:

As used here, the term plant includes plant cells in planta and plant cells and plant protoplasts in culture, plant cell tissue cultures from which corn plants can be regenerated, aggregations of plant cells such as is present as a disorganized mass in a callus, plant clumps, and plant cells that are intact in plants or parts of plants, such as embryos, pollen, flowers, kernels, ears, cobs, leaves, shoots, seeds, fruit, husks, stalks, roots, root tips, anthers, silk and the like.

By "selectable marker genes and reporter genes" is meant a DNA sequence coding for a phenotypical trait by means of which transformed cells may be selected from untransformed cells.

By "transcription termination sequences" is meant any nucleic acid sequence which determines the position of the 3' end of a transcript. Transcription termination sequences include polyadenylation sites. By "transformation" is meant the act of causing a cell to contain a DNA sequence which did not originate in that cell. By "operably linked" is meant the chemical fusion of two fragments of DNA in a proper orientation and reading frame to be transcribed into functional RNA.

The preferred type of corn species is obtained from the American Type Culture Collection (ATCC), Rockville, Md. 20852 U.S.A. For example, a tissue culture of regenerable cells of a plant of inbred corn line (for example, public inbred lines H99, Pa91) that is highly regenerable from calli, as described in Hodges et al., Bio./Technol. 4:219-223 (1986), is preferred.

Applications

Normally, extraction of gelatin from skins and bones is an expensive process and contributes at least 20% of the cost of producing gelatin. As contemplated herein, it is not necessary to isolate, extract or purify the collagen compound from the corn plant in an additional, separate process; the wet-milling process which is typically used to extract corn starch and corn gluten may be used to extract the collagen compound. The resulting gelatin may be used in the production of cultured dairy products such as yogurt, ice cream, pudding, sour cream and other milk-based products. This has the advantage of utilizing corn for both corn starch and gelatin. The resultant gelatin is usable in the food industry with the added benefit of a low-cost, vegetarian (non-dairy) kosher product. Corn is the preferred plant as it has the advantages of low raw material cost (in the range of $2.00 to $2.50 per bushel or 56 pounds of dry kernels) and an existing wet milling extraction process.

The resultant gelatin may also be used in such applications as photography, adhesives, pharmaceutical and medical uses, paper and textile making and other uses by conventional methods. The recovered collagen may be used in medical and therapeutic procedures and for cosmetics applications.

There are several advantages to using transgenic plants as bioreactors over bacteria, yeast or isogenic cattle. While the estimated cost of generating transgenic plants may be high initially, the yield and overall cost of the resultant collagen and gelatin will be very low. Collagen or gelatin may be extracted from transgenic corn plants using existing wet-milling technology. In contrast, while collagen or gelatin produced in milk of isogenic cattle may be used directly in the production of dairy foods, other uses for the collagen or gelatin requires extraction of the collagen or gelatin from the milk. This extraction process is an additional, separate step which is not part of milk processing as currently practiced. In practice, bacteria and yeast bioreactors can be very expensive to produce foreign proteins.

The use of transgenic plants has other applications as well. With appropriate gene constructs the expression of the inserted genes in transgenic plants may be controlled in a tissue-specific and in a differentiation-specific manner. Plants may be altered not only to produce a collagen compound but also to obtain an "enhanced gelatin effect" when the collagen compound is produced in a plant which is also the source of a food gum such as corn starch, guar gum, locust bean gum, etc. By "enhanced gelatin effect" is meant that there are additional, noticeable effects on gelling properties as a result of the synergistic interaction of the collagen compound from which gelatin is derived and the food gum which is produced by

the same plant. Although the primary effect is that of gelling, it is understood that this can affect the overall properties and cause general improvement in the resulting gel. The collagen and food gum may either interact during growth of the plant or alternatively, where the collagen and the food gum are present in different cellular compartments of the plant, the enhanced effect is initiated during processing of the crop, when collagen/gelatin and the food gum are brought together. Technologies associated with milling of food gum producing crops and starch producing crops are adapted to harvest collagen, gelatin or a gelatin-food gum combination.

Examples of suitable monocotyledonous plants into which the gene construct may be inserted include corn (*Zea mays* Linnaeus), rice, wheat, barley, oats, millet, sorghum amaranth, onion. asparagus and sugar cane. Suitable dicotyledonous plants include alfalfa, soybean, cotton. clover, sugarbeet, sunflower, carrot, celery, cabbage, cabbage, broccoli, brussel sprouts. radish, rapeseed, cucumber, pepper, canola, bean, lettuce, cauliflower, spinach, artichoke, pea, okra, squash, kale, collard greens, potato, tobacco, tomato, tea and coffee and the like.

Other suitable plants for use in this invention are plants from which food gums are derived. Food gums or hydrocolloids are water-soluble or -dispersible polysaccharides (glycans) and their derivatives and gelatin (which is a protein rather than a polysaccharide). Gums are useful due to their capacity to thicken or gel aqueous systems at low concentration. Gums are utilized as binders, bodying agents, bulking agents, crystallization inhibitors, clarifying agents. cloud agents, emulsifying agents, emulsion stabilizers, encapsulating agents, film formers, flocculating agents, foam stabilizers, gelling materials, mold release agents, protective colloids, suspending agents, suspension stabilizers, swelling agents, syneresis inhibitors, texturing agents and whipping agents, in coatings and for water absorption and binding.

Polysaccharide food gums include seed gums such as corn starch, guar gum and locust bean gum; tuber and root gums such as potato starch, tapioca starch and konjac mannan. Gums such as algins, carrageenans and agar are seaweed extracts.

Other suitable plants useful in the invention are of the family Leguminosae and include the guar plant, *Cyanoposis tetragonolobus*, the source of guar gum, and the locust or carob tree, *Ceratonia siliqua*, from which locust bean gum is derived. Also suitable are seaweeds including Irish moss *(Chondrus crispus)* from which the polysaccharide gum

carrageenan is extracted, and giant kelp, *Macrocytis pyrifera*, which is the principal source of commercial alginate. Other related brown seaweeds which are also suitable are rockweed (*Ascophyllum nodosum*) and the several *Laminaria* species, from which alginates are recovered. Many seaweeds belonging to the *Gracillaria, Gelidium*, and *Acanthopeltis* groups yield agar on extraction and these plants are also suitable in the method of the invention.

Food chemists have found that combining two food gums, such as κ-carrageenan and locust bean gum, yields a desirable effect on the food being prepared. A more elastic gel with markedly greater gel strength, viscosity, gel elasticity and less syneresis is produced. The combination of κ-carrageenan with konjac mannan also produces desirable results. At high concentrations, carrageenan increases the gel strength of guaran, but at low concentrations it produces only an increase in viscosity. The combination of two food gums has never before been accomplished in plants.

It is advantageous to insert the gene construct of the invention in plants which produce food gums to achieve an enhanced gelatin effect due to the interaction of the food gum and the collagen compound. It should be understood that the present invention is not limited to the plants described herein and other plants may be similarly employed.

Collagen-Gelatin Transition

The typical vertebrate collagen molecule is a rigid rod about 3000 Å long and 15 Å in diameter, with a particle weight of about 3×10^5 g/mole. It is composed of three polypeptide chains of approximately equal length, with an average weight per chain of about 1×10^5 g/mole. The triple helix of collagen I is a heterotriplex containing the products of two different collagen-encoding genes and is designated $[\alpha_1(I)]_2\ \alpha_2(I)$. Collagen I triplexes contain two protein chains encoded by the Col1A1 gene and one protein chain encoded by the Col1A2 gene. When heated above a critical temperature or as a consequence of changes in a solvent environment that lower the critical temperature below the experimental temperature, the rigid three-stranded collagen molecule collapses to a mixture of random-coil components collectively termed gelatin. This treatment results in the separation of non-covalently cross-linked molecules into three single-chain random-coil units, termed the α-components.

In mammals, fibroblasts typically synthesize and secrete collagen. The following co-translational and post-translational modifications occur when collagen is produced in fibroblasts: cleavage of "signal" peptides at the N-termini of the chains, hydroxylation of the Y-position proline and lysine residues, hydroxylation of a few X-position proline residues,

addition of galactose or addition of galactose and then glucose, to some of the hydroxylysine residues, addition of a mannose-rich oligosaccharide to the C propeptides, association of the C-terminal propeptides, and finally formation of both intrachain and interchain disulfide bonds in the propeptides. After secretion of procollagen from fibroblasts, the N propeptides are cleaved by a procollagen N proteinase and the C propeptides are cleaved by a separate procollagen C proteinase. The collagen then self-assembles into fibrils. Lysyl oxidase converts some lysine and hydroxylysine residues to aldehyde derivatives that form cross-links with similar residues in adjacent molecules as disclosed by Prockop, DJ et al., New England J. Med. 311:376-386 (1984).

Most proteins require extensive post-translational modification in order to exhibit full biological activity. In a preferred embodiment, post-translational modifications and biological activity of the compound is not a concern. The posttranslational events of the protein of interest, collagen, may be important but not critical to its capacity to gel. As an objective of the invention is the production of gelatin which has a random coil configuration, the presence of appropriate enzymes to ensure proper assembly of the collagen into triplexes is immaterial. So long as the collagen compound produced in the plant has the ability to gel when recovered, the structure, nature and biological activity of the collagen compound is not critical. Only a limited region of the collagen protein is required for the resulting gelatin to display a gelling effect. G. Stainsby in The Science and Technology of Gelatin, edited by A.G. Ward and A. Courts, Academic Press 1977, pg. 196-199. Accordingly, the collagen compound may be a fragment of a protein, such as a fragment of procollagen or collagen. Specifically, the collagen compound may comprise the COLIA1 chain, the COLIA2 chain, both the COLIA1 and COLIA2 chains or appropriate portions thereof. In one embodiment, the collagen compound may be collagen I alpha 1 which has the ability to gel as a homotrimer. Of course, gelatin derived from classical bovine collagen I which is comprised of two chains of COL1A1 and one chain of COL1A2 will exhibit a higher Bloom strength than other gelatins, so that classical bovine collagen I may be the preferred collagen compound for some applications.

Gene constructs

The invention also contemplates a gene construct which comprises a DNA sequence coding for the collagen compound operably linked upstream (5') to a promoter sequence capable of initiating and directing transcription in plant cells of said DNA sequence. The

gene construct is integrated into the plant genome such that upon transformation the plant cells express the collagen compound. Transgenic plants are regenerated from the plant cells which have been transformed and grown for a time and under conditions sufficient to permit expression of the collagen compound.

The invention is also directed to seeds obtained by growing the transgenic collagen compound-producing plants, collagen produced from plants, gelatin produced from plants and food products which include gelatin produced from plants.

The proper regulatory signals must be present in the proper location with respect to the gene for the newly inserted collagen gene to express the protein for which it codes in the plant cell. These regulatory signals include a promoter sequence that directs the cellular machinery to produce RNA and a polyadenylation sequence which is a non-translated region that terminates transcription in plant cells and causes the addition of polyadenylate nucleotides to the 3' end of the RNA to stabilize the RNA in the cytoplasm for subsequent translation of the RNA to produce protein.

The promoter may be any constitutive, inducible, tissue or organ specific, or developmental stage specific promoter which can be expressed in the plant cell of choice. Constitutive promoters, such as the 35S promoter of cauliflower mosaic virus, which direct RNA production in many or all tissues of a plant and during most stages of development when integrated into the genome of transgenic plants are preferred. In particular, the 35S promoter from the cauliflower mosaic virus (CaMV35S), which has been shown to be the strongest constitutive promoter known in plants and which confers expression in both dicots and monocots, is preferred. Odell et al., Nature, 313:810-812 (1985); Jensen et al., Nature, 321:669-674 (1986); Jefferson et al., EMBO J., 6:3901-3907 (1987); Kay et al., Science, 236:1299-1302 (1987); Sanders, et al., Nucleic Acids Research, 4:1543-1558 (1987). In particular, a chimeric promoter with doubled CaMV enhancer elements, referred to herein as a CaMV35S doubled enhancer promoter, is preferred.

It is critical that the gene construct consisting of at least a promoter and the DNA sequence coding for the collagen gene integrates into the genome of the corn so that it is stably inherited by progeny of the transgenic corn. Stable transformation comprises the chromosomal integration, including incorporation into plastid chromosomes, of the introduced DNA so that the integrated gene sequences are passed on to progeny of the transformed plant. Stably transformed cells must also be capable of regenerating fertile,

transgenic plants. Transient transformation, in contrast, does not generate transgenic plants as the introduced DNA is eventually lost.

Electroporation is the presently preferred method of introducing foreign DNA into corn. Fromm et al. Nature, 319:791-793 (1986); Jones et al., Plant Mol. Biol., 13, 501 (1989); Yang et al., Plant Cell Reports, 7, 421 (1988); D'Halluin et al., The Plant Cell R:1495-1505 (1992) and Krzyzek et al., U.S. Patent No. 5,384,253.

Thus, the invention contemplates cloning the appropriate bovine collagen genes from genomic DNA or from messenger RNA as cDNA. A cDNA construct will not contain all the introns while the genomic DNA will. Preferably, bovine collagen type I (either the α1 or α2 chains, or both chains or fragments thereof) is cloned. Appropriate DNA sequences containing the bovine collagen compound gene or fragments thereof linked to a plant specific promoter such as the CaMV35S promoter are constructed and used to transform corn (maize). Constructs are made using specific restriction enzymes which recognize certain sequences of bases on the DNA strand and cut where those sequences appear. Ligases are enzymes which rejoin DNA segments. The cut DNA is ligated (joined) to a DNA vector which allows the gene of interest to become incorporated into the plant genome. The vector may contain viral and/or plasmid features. Heterozygous transgenic plants that synthesize the collagen compound are generated. Transformation occurs when the gene carried by the vector is incorporated into the DNA of the plant where it initiates production of the desired collagen compound. Using backcrossing techniques well known to those of ordinary skill in the art, homozygous maize for commercial planting is then generated. Transformed plants are grown and either pollinated with the same transformed strain or different strains. The resulting progeny expressing the collagen compound is then identified. Two or more generations may be grown to insure that the collagen compound is stably maintained and inherited and then seeds are harvested for use to provide plants expressing the desired collagen compound. Similar procedures are followed to obtain collagen compound of human or fish origin.

Cloning The Bovine Collagen I Genes

Overview

The entire complement of genes in an organism that encodes proteins needed for development and life is called the genome. In mammals, the genes are organized on chromosomes with each chromosome being a single long molecule of double stranded DNA. The DNA in the chromosomes functions as a blueprint for proteins. Many genes are encoded

as a single uninterrupted sequence of nucleotides. Some genes are arranged in segments with coding regions (exons) interrupted by non-coding regions (introns). When a particular protein is needed, the code to synthesize the protein is transferred from the DNA into a molecule of messenger RNA (mRNA) by a process called transcription. The 5' end of the mRNA encodes the protein's amino terminus (beginning with the first exon) and the 3' end encodes the carboxyl terminus. Nucleotides preceding the first exon are referred to as being "upstream" from the gene and nucleotides after the last exon are "downstream" from the gene. RNA transcription is initiated at a specific upstream DNA sequence called a promoter. Transcription rate may be accelerated by the presence of sequences in the DNA called enhancer elements and these may be upstream or downstream of the promoter. After transcription, the mRNA may be further processed by splicing before leaving the nucleus. The completed mRNA enters the cytoplasm where it encounters ribosomes which translate the codes contained in the mRNA into the appropriate protein.

Where not specified, recombinant DNA procedures follow the methods of Sambrook, J., Fritsch, E.F., and Maniatis, T., Molecular Cloning, A Laboratory Manual, Second Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), incorporated herein by reference. Restriction enzymes and T4 ligase are obtained from commercial sources, such as New England Biolabs of Beverly, Massachusetts, and used according to the manufacturer's recommendations.

Cloning the human collagen gene

The gene encoding the alpha I polypeptide chain of human type I collagen (COL1A1) has been cloned as disclosed by Barsh, G.S. et al, Journal of Biological Chemistry 259(23):14906-14913 (1984), incorporated herein by reference. It spans a large area (18,000 base pairs) and is composed of multiple exons. In addition to the upstream promoter, the COL1A1 gene transcription may also be regulated by sequences within the first intron (noncoding region between the first and second exons) as disclosed by Bornstein, P., Proc. Natl. Acad. Sci, USA 84:8869-8873 (1987).

Cloning the genomic bovine collagen I alpha I gene and bovine collagen I alpha II gene

A procedure similar to that for cloning human collagen type I alpha 1 is used for cloning bovine collagen. Specifically, a bovine genomic library is created by isolating DNA from bovine fibroblasts, cutting it with appropriate restriction enzymes to generate large fragments of DNA and cloning them into a cosmid vector, using standard techniques such as

disclosed in Sambrook and Maniatis, Molecular Cloning, Second Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). Large segments of DNA (30-42 kilobases) are cloned into a single cosmid. The resulting library is screened with cDNA probes made by restriction digestion of the human collagen I alpha 1 cDNA clone in the conventional way disclosed by Tromp, G., Biochemical Journal 253:919-922 (1988), incorporated herein by reference, to identify and isolate the bovine collagen I alpha I gene. The cDNA is preferred for screening instead of the genomic human clone because the cDNA contains only exons. The exons of collagen are highly conserved between species whereas introns may not be highly conserved as disclosed by Bernard, M.P., Biochemistry, 22(22):5213-5223 (1983). The cDNA probes allow detection of the exons within the bovine genomic fragments contained in the bovine genomic library. The library is screened with a probe which encodes the 5' region of the human collagen gene and subsequently positive clones are then screened with human probes encoding the far 3' region of the collagen gene. The double screening may result in a single clone containing the entire bovine COL1A1 gene. If a full length clone is not contained within a single cosmid, the library may be rescreened with the 3' probe and positive clones analyzed for sequences which overlap the clones "pulled" from the library with the 5' probe. Restriction enzyme analysis of each resulting clone enables creation of a hybrid clone encoding the entire gene by splicing into the overlapping intron regions. Furthermore, clones which hybridize with the 5' probe but not the 3' probe may be used to identify the bovine collagen promoter and enhancer elements. These techniques result in (a) a full length genomic clone encoding the bovine COL1Al gene and (b) a clone containing the collagen transcriptional promoter and enhancer elements. These recombinant constructs are then used to create clones to be used for gene constructs to generate transgenic corn. The same strategy is followed to clone the bovine collagen type I alpha 2 gene. The cloned human COL1A2 cDNA is used in the conventional manner disclosed by Kuivaniemi, et. al., Biochemical Journal 525:633-640 (1988), incorporated herein by reference, to create 5' and 3' probes for the isolation of a bovine collagen type I alpha 2 full length genomic clone and regulatory elements.

Cloning the bovine cDNA encoding the bovine type I alpha 1 collagen polypeptide and bovine type I alpha 2 collagen polypeptide

cDNA libraries are created from mRNA transcripts and do not contain the intronic sequences of the genomic gene. Many genomic genes are silent in differentiated or

specialized cells. The active genes are transcribed and proteins synthesized from the messages. Therefore, tissue specific or differentiation specific clones can be generated from the mRNAs being synthesized at the time of RNA extraction. RNA is extracted from specialized bovine cells which synthesize collagen type I and double strand cDNA is synthesized using standard techniques to create a DNA bovine library encoding for bovine type I alpha 1 collagen. The average size mRNA in a cell is usually around 2 kilobases. However, the human collagen type I (and other collagen) message is quite large (4-5 kilobases) and bovine collagen type I is similar in size. While most plasmid vectors used in cloning are able to handle inserts of this size, it may be advantageous to clone into lambda phage vectors which can accept inserts as large as 10 kilobases without replication problems. Plasmids containing smaller inserts will outgrow those containing large inserts and may make screening more difficult. It may be necessary to enrich for large mRNAs by size fractionation over sucrose density gradients. After enrichment the resulting RNAs are reverse transcribed into cDNA and ligated into the vector of choice. This results in a library enriched for the bovine collagen I transcript. The library is screened with the human collagen alpha 1 cDNA probe as discussed above with respect to a genomic library. Positive clones are sequenced to determine whether they are full length. If no full length clones are obtained, clones which contain areas of overlap may be spliced together to create a full length bovine cDNA. This results in a clone encoding bovine type I alpha 1 collagen. This cDNA clone encodes for a procollagen protein. This clone may be used to create gene constructs by ligating it with the appropriate promoter and enhancers for generating transgenic corn.

The collagen type I alpha 2 gene is isolated in a similar manner using the human collagen type I alpha 2 cDNA as a probe as discussed above with respect to a genomic library to screen the cDNA bovine collagen type I alpha 2 library. In all other respects, the process for collagen type I alpha 2 is the same as for collagen type I alpha 1.

Cloning any collagen compound gene

Similar processes, as outlined above, may be followed for cloning any other collagen compound gene such as collagen II through collagen XIX and fragments thereof, of human, animal or fish origin. The resulting clones are used for DNA constructs to generate transgenic corn. It is understood that new probes for use in cloning collagen and new methods of cloning different types of collagen may be developed for use in the invention.

Creation of clones for generating transgenic plants

Recombinant collagen gene constructs are used to generate transgenic plants which are able to produce procollagen, the collagen I alpha 1 and/or collagen I alpha 2 chains or fragments thereof in their cells. Either a constitutive promoter, such as the CaMV35S promoter, or a desired tissue specific or differentiation specific promoter is then ligated to the gene encoding the desired collagen compound using standard techniques now common in the art. A transcription termination sequence is ligated downstream of the structural gene to provide for efficient termination.

Specific Examples of Gene Constructs

Two different types of gene constructs shown in Figs. 1 and 2 and described below are preferred. The preferred gene constructs are integrative (integrate into host DNA).

Constructs using the cDNA bovine clone

The cloned bovine cDNA prepared as described above is put under the regulatory control of the cauliflower mosaic virus 35S promoter utilizing conventional genetic manipulation techniques.

Constructs using the cDNA human, animal or fish clone

The cloned human, animal or fish cDNA prepared as described above is put under the regulatory control of the cauliflower mosaic virus 35S promoter utilizing conventional genetic manipulation techniques

Generating transformed plant cells

The preferred gene constructs for producing procollagen or collagen to be used to produce gelatin are as follows:

Referring to Fig. 1, the preferred gene construct comprises a CaMV35S doubled enhancer promoter fused to the collagen compound gene, which may be the cloned bovine collagen type I alpha 1 gene or the collagen type I alpha 2 gene or the procollagen genes or any member of the collagen compound gene family as defined above 5' to the start codon for protein translation. A transcription termination sequence, including a polyadenylation signal, from the nopaline synthase or octopine synthase genes or from CaMV is fused to the 3' end of the collagen compound gene to ensure cessation of translation. After transcription termination, polyadenylic acid "tails" are added to the 3' end of mRNA precursor for message stability. This results in a first gene fragment. A second gene fragment carrying the neomycin resistance gene under the regulatory control of a second CaMV35S promoter and a transcription termination sequence are fused to the 3' end of the first gene fragment. The

resulting gene construct is cloned into a bacteria vector in toto in order to grow the construct for DNA purification. The gene construct is then introduced into a plant for integration into the host chromosome as discussed below.

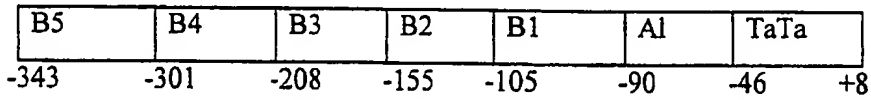
Other elements such as leader sequences, introns, enhancers, polyadenylation sequences and the like may optionally be included in the foreign DNA to improve expression or functioning of the introduced DNA in the plant by affecting transcription, stability of the mRNA, etc. For example, insertion of the maize alcohol dehydrogenase 1 intron 1 (Adh-l) (Callis et al., Genes and Develop., 1, 1183 (1987)) or the rice actin 1 intron 1 (Act-1) sequences (McElroy et al., Mol. Gen. Genet. 231:150-160 (1991)), between a promoter and a coding sequence in a particular recombinant DNA construct leads to a 10-65 fold increase in production of a reporter enzyme in transgenic maize plants. (Callis et al., supra). However, even without inclusion of an intron or enhancer element in the gene construct, sufficient expression for a selectable marker to facilitate identification and selection of transformed cells may often be obtained. Klein et al., Plant Physiol., 91, 440 (1989). Gene constructs containing the Adh-l or Act-1 enhancer elements contained within the introns are preferred to increase expression of the collagen compound gene in maize cells. Referring to Fig. 2, the rice actin 1 intron 1 or corn alcohol dehydrogenase intron 1 is inserted between the CaMV35S doubled enhancer promoter and the collagen compound gene. The Act 1 intron 1 gene is obtained by digestion of plasmid pBCG-A4 with XbaI and NcoI as disclosed in McElroy et al., Mol. Gen. Genet. 231:150-160 (1991). The Adh 1 intron 1 sequence is obtained by restriction digest of plasmid pCI_1GusN with XbaI and SmaI as disclosed by McElroy et al., Mol. Gen. Genet. 231:150-160 (1991).

Constructs using the cDNA bovine clone

This construct encodes the collagen alpha l and collagen alpha 2 procollagen genes. In one embodiment, the cDNAs encoding procollagen are cut with appropriate restriction enzymes to remove the nucleic acids residues which encode procollagen amino and carboxyl terminal amino acids. This latter construct is referred to as bovine collagen cDNA. These terminal amino acids are needed for correct association of two collagen type I alpha 1 and one collagen type I alpha 2 procollagen chains to form the procollagen triple helix. The terminal amino acids are cleaved when the procollagen molecule is secreted from cells and this cleaved molecule is referred to as collagen. In this embodiment of the invention, it may not

be necessary for procollagen to be secreted by the plant cells as long as the protein may be stored without degradation.

The CaMV35S promoter used herein is described in Benfey and Chua, Science 250:959-966 (1990). Analysis of the CaMV 35S promoter indicates two domains, domain A from nucleotide -90 to +8, and domain B from nucleotide -343 to -90, and five subdomains, B1 through B5. whose end points are indicated below, located upstream of the Tata region (-46 to +8) of the CaMV35S promoter.



The promoter sequences used are derived from plasmid pKM794 described in Omirulleh et al., Plant Molecular Biology, 21:415-428 (1993) as attributed to Drs. E. Fejes and F. Nagy (unpublished). Plasmid pKM794 contains a CaMV doubled enhancer promoter sequence fused to the GUS gene with NOS terminator. The doubled enhancer promoter construct of plasmid pKM794 contains a tandem repeat of domains B3 to A1 (nucleotides -208 to -46) inserted upstream of the promoter domains A1 and Tata (nucleotides -90 to +8) of the CaMV35S promoter. This entire doubled enhancer/promoter unit is excised from plasmid pKM794 by cleaving with Hind III/Bam HI.

Octopine Ti plasmids carry an ocs gene which encodes octopine synthase (lysopine dehydrogenase). Koncz et al., EMBO J. 2:1597-1603 (1983) provides a functional analysis of ocs. Nopaline Ti plasmids encode the nopaline synthase gene (nos) (sequenced by Depicker et al.. J. Mol. App. Gen. 1:561-573 (1982). A functional analysis of nos is provided by Shaw et al., Nucl. Acids Res. 12:7831-7846 (1984).

The CaMV35S doubled enhancer promoter sequence is fused to the bovine collagen/procollagen type I alpha I or bovine collagen/procollagen type I alpha 2 or human collagen/procollagen clone using established molecular biology techniques, following Sambrook, J., Fritsch, E.F., and Maniatis, T., Molecular Cloning, A Laboratory Manual, Second Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

A transcription termination sequence including a polyadenylation signal is fused to the 3' end of the CaMV35S doubled enhancer promoter sequence-collagen compound gene. The transcription termination sequence may be obtained from the same source as the promoter (i.e., from CaMV) or may be obtained from a different source. Omirulleh et al.,

Plant Mol. Bio. 21:415-428 (1993) discloses a 260 base pair sequence containing a 3' transcription terminator sequence of the nopaline synthase gene in plasmid pKM794. This 260 base pair sequence is derived from restriction digest of plasmid pKM794 with SacI and EcoRI and is ligated to the 3' end of the doubled enhancer promoter sequence-collagen compound gene using techniques as disclosed by Maniatis. Alternatively, restriction digest of plasmid pNA2G disclosed by Omirulleh et al., Plant Mol. Bio. 21:415-428 (1993) with EcoRI and PstI releases the CaMV35S terminator/polyadenylation fragment, which is then ligated to the 3' end of the doubled enhancer promoter sequence-collagen compound gene as discussed above. Another vector construct, pDE108, disclosed in D'Halluin, The Plant Cell 4:1495-1505 (1992), uses an octopine synthase termination/polyadenylation nucleotide sequence which is released from pDE108 by cleavage with NcoI and Hind III and subsequently ligated to the 3' end of the doubled enhancer promoter sequence-collagen compound gene.

The foreign DNA coding for a collagen compound to be introduced into the plant preferably contains a selectable marker or a reporter gene, or both, effective in plant cells to aid in isolation of transformed cells and to promote identification and selection of transformed cells. The selectable marker may alternatively be carried on a separate piece of DNA and used in a co-transformation procedure. The selectable marker and/or reporter genes are flanked with appropriate regulatory sequences to enable expression in plants. Selectable markers useful in the invention include genes encoding antibiotic and herbicide resistance such as are well known in the art. A preferred drug resistance marker is the gene whose expression results in neomycin resistance. After transforming the plant cells. those cells having the gene construct will be identified by their ability to grow on a medium containing the particular antibiotic.

Reporter genes encode easily assayable marker proteins whose expression is manifested by some easily detectable property, e.g., change in phenotype or enzyme activity. Reporter genes are not present in or expressed by the recipient cell or tissue. They are used to determine whether a specific foreign DNA sequence can transform a plant cell. Preferred reporter genes include the luciferase genes from firefly *Photinus pyramis*. After the foreign DNA is introduced into the recipient cells, cells are assayed for expression of the reporter gene.

The first gene fragment comprising the doubled enhancer promoter sequence-collagen compound gene-transcription termination sequence is fused to an EcoRI/HindIII fragment of plasmid pDE108 containing the entire sequence of the CaMV promoter - neomycin resistance gene - octopine synthase gene terminator region (3' ocs) described by D'Halluin et al., The Plant Cell 4:1495-1505 (1992). This entire new construct is cloned into a bacterial vector such as pBluescript (Stratagene, California) at an appropriate restriction site(s) for growth and isolation of the DNA. Before transformation of plant cells, the gene construct is excised from the bacterial vector with appropriate restriction enzymes and purified by agarose gel electrophoresis to remove any bacterial vector DNA. The resulting gene construct will be used for transformation.

Corn Tissue Culture

Unless otherwise specified, corn tissue culture procedures are as described in Green and Rhodes, "Plant Regeneration in Tissue Culture of Maize", Maize for Biological Research (Plant Molecular Biology Association, Charlottesville, Va. 1982, at 367-372) and in Duncan, et al., "The Production of Callus Capable of Plant Regeneration From Immature Embryos of Numerous Zea Mays Genotypes", 165 Planta 322-332 (1985). Methods of preparing and maintaining calli from maize tissue are described in R. Phillips et al., Corn and Corn Improvement, Agronomy Society of America (3d ed., 1988) at pages 345-387.

Transformation of maize cells

Methods to introduce the collagen compound gene into plants include the direct transfer of DNA by microinjection, electroporation, DNA entry aided by polyethylene glycol, injection of plant tissue with *Agrobacterium tumefaciens* engineered to carry foreign DNA into plants, liposome fusion, use of a particle gun to inject DNA-coated microprojectiles and the like. There are advantages and disadvantages to each of these methods. A particular method of introducing the gene construct into a particular plant species may not be the most effective for another plant species. For instance, *Agrobacterium* mediated transformation has been used successfully to transform dicotoleydonous plants but its utility for monocot transformation is limited. Electroporation is the presently preferred method for transformation of corn so that the foreign DNA is stably integrated in the corn plant genome and inherited by progeny of the transformed corn plants. D'Halluin et al., The Plant Cell, 4:1495-1505 (1992). U.S. Patent No. 5,384,253 to Krzyzek et al, incorporated herein by reference, discloses a preferred method of transforming corn via electroporation.

To successfully produce fertile transgenic plants by electroporation, target cells are treated to render them competent for uptake of foreign DNA without significantly reducing viability. These "transformation-competent" cells incorporate the foreign DNA at appropriately high frequencies to stably transform a sufficient number of cells. The transformed cells are capable of maintaining cell division and regenerative capacity throughout the selection processes necessary to confirm and identify stably transformed cells. The transformed regenerated plants are capable of passing on the introduced DNA to progeny so that the progeny express the introduced DNA.

Three types of cells are preferred for use in transformation and subsequent production of transgenic maize: (a) immature zygotes isolated from ears, (b) type I callus produced by culture of immature zygotes, and (c) suspension cultures of type II callus generated from the type I callus cultures.

Preparation of callus and suspension cell cultures

Regenerable maize suspension cell cultures may be derived from a number of plant tissues. Preferably, the cell cultures are derived from calli generated from immature maize embryos which are removed from the kernels of an ear when the embryos are about 1-3 mm in length (about 9-14 days after pollination). Embryos are aseptically isolated and placed on nutrient agar initiation/maintenance media with the embryo root/shoot axis down (scutellum up). The initiation maintenance media (i.e., F medium, obtained from Gibco, Grand Island, New York) consists of N6 basal media (Chih-ching, Proceedings of Symposium on Plant Tissue Culture, May 25-30, 1978. Science Press, Peking, pp. 43-50) with 2% (w/v) sucrose, 1.5 mg/liter 2,4-dichlorophenoxyacetic acid, 6 mM proline, 200 mg/l casein hydrolysate and 0.25% Gelrite (Kelco, Inc., San Diego). Callus tissue (type I) appears and grows from the scutellum after several days to a few weeks. The callus tissue from the scutellum is evaluated for friable consistency and the presence of well-defined somatic embryos which would indicate that the cultures are regenerable under proper conditions. Tissue is of "friable consistency" when it is easily dispersed without causing injury to the cells. Tissue meeting this definition is transferred to fresh media and subcultured on a routine basis about every two weeks.

After about 4-6 months, the established callus cultures is referred to as type II callus and is transferred to liquid growth media. Methods for producing regenerable suspension cell cultures are described by C. E. Green et al. in Maize for Biological Research, Plant Molec.

Biol. Assoc. (1982) at pages 367-372; R. Phillips et al., Corn and Corn Improvement, Agronomy Soc. Amer., (3d ed., 1988) at pages 345-381; and I. Vasil, Cell Culture and Somatic Cell Genetics of Plants, Vol. I, Laboratory Procedures and Their Applications, Academic Press (1984) at pages 152-158. The liquid growth media for suspension cell cultures is typically of similar formulation to F media with ABA (abscisic acid) (10.sup -7 M) added to augment regenerative capacity and enhance culture vitality. The callus is preferably not sieved prior to introduction into the liquid growth media. The cultures in liquid media are subcultured as appropriate to maintain active growth.

Preparation of Transformation-Competent Cells

"Transformation-competent cells", defined as cells having an increased ability to take up, express and integrate foreign DNA by electroporation as compared to untreated cells, are prepared by partially enzymatically degrading the cell walls of the cells by the controlled exposure of cells to one or more pectin-degrading enzymes. Hydrolysis of the pectin component of the cell wall is believed to increase the cell's permeability to foreign DNA while preserving the viability of the cell. In contrast, previous production of transgenic dicots has been accomplished by completely enzymatically degrading the cell walls of type II callus thereby creating a type of competent cells termed protoplasts. However, electroporation of monocot protoplasts (i.e., maize) results in extremely low transformation rates and often phenotypically abnormal plants (i.e., reduced size, seed number and viability). Krzyzek et al., supra; D'Halluin et al., The Plant Cell 4:1495-1505 (1992).

Transformation competent cells prepared as disclosed in Krzyzek et al., U.S. Patent No. 5,384,253, are morphologically and physiologically different from protoplasts in a number of ways including the shape and conformation. Transformation-competent cells retain the out-of-round shape of callus cells and consist of stable multicell clumps in culture while protoplasts are spherical and unless they reversibly agglutinate, do not clump. As portions of the cell wall are still intact, transformation-competent cells are stainable with Tinapol BPOT (Ciba-Geigy), a cellulose-specific stain. No staining is observed with protoplasts as the cells wall is completely degraded.

Polysaccharidase enzymes such as one or more pectin degrading enzymes are used to break down part of the maize cell walls. The term "pectin degrading enzyme" includes enzymes that catalyze the breakdown of pectin and pectin subunits. Examples of pectin-degrading enzymes include endopectin lyase, pectin lyase, pectolyase,

endopolygalacturonase, and polygalacturonase, as well as pectinase. Other enzymes such as xylanase, cellulase, hemicellulase, driselase, transeliminase, or macerozyme, may also be used in combination with pectin-degrading enzymes. Pectolyase (Sigma Chem. Co., St. Louis, Mo.), a combination of endopectin lyase and endopolygalacturonase, is preferably used to prepare transformation-competent cells. It is also available from Seishin Pharmaceutical Co. (Tokyo, Japan) as "Pectolyase Y-23".

Enzymatic treatment is carried out for a time sufficient to partially break down the maize cell walls without adversely affecting the viability, mitotic activity or regenerative capacity of the cells as disclosed by Krzyzek et al., supra. For a dilute solution of enzyme(s) (0.1-1%) digestion time ranges from about 0.75 to 3.0 hours, preferably from about 1.5-2.0 hours for a packed volume of about 1-2 ml of maize cells/5 ml enzyme buffer at about room temperature. Optionally, the cultured maize cells are declumped by sieving or filtering to increase the cell wall surface area exposed to enzymatic action. Following partial degradation of the cells walls, the cells are washed with buffer in an amount sufficient to substantially remove residual enzymes.

Electroporation of Suspension Cultures

The gene construct of the invention is introduced into cultures of transformation-competent maize cells prepared as described above by electroporation to obtain transformed cells which are regenerable into fertile transgenic maize plants. Potter et al., PNAS USA, 81, 7161 (1984) describes a suitable electroporation apparatus for use in the invention. Such devices typically consist of an electronic device including a capacitor. The capacitor is charged and incorporated into a circuit in series with the cells to be electroporated in an electroporation buffer. The capacitor is then discharged so deliver a current pulse to the cells. The waveform, pulse length and number of pulses delivered and pulse field conditions may be varied to optimize cell viability and levels of expression of the transformed cells.

Electroporation of transformation-competent cells is preferably performed within about 45 minutes of their preparation, although transformation-competent cells left for as long as 3 hours before electroporation may be transformed at acceptable frequencies. Electroporation is performed in an electroporation chamber in the presence of electroporation buffer at room temperature (20° - 30° C). K.J. Paite, Plant Cell Reports, 4, 274 (1985). The formulation, osmolarity, pH, concentration and ionic composition and other parameters of the electroporation buffer are adjusted to optimize cell viability and transformation.

The electroporation buffer must be compatible with, and must not be toxic or otherwise adversely affect the cells to be transformed. The electoporation buffer will generally contain the gene construct, a buffering agent to maintain the pH of the electroporation buffer between about 7 and 7.7, preferably at about 7.5, and an osmoticum. Suitable buffering agents include HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) and sodium phosphate, and mixtures thereof. The osmoticum in the electroporation buffer is a compound(s) that helps maintain an osmotic balance between the extracellular medium and the interior of the transformation-competent maize cells, so cell viability is preserved. Preferred osmotica are sugars such as fructose, sucrose and the like and polyols, preferably C_2 - C_6 polyols such as mannitol, sorbitol, glycerol and the like. The optimal concentration of the osmoticum is approximately 0.3-0.5M. The use of mannitol was found to lead to a consistently higher rate of transformation than using either sorbitol or sucrose.

The gene construct present in the electroporation buffer may be in supercoiled or linear form. In preferred embodiments, linear double-stranded DNA, e.g., from recombinant plasmids, is employed. Although concentrations as low as about l μg/ml per 1.0 ml of electroporation buffer may be used, it is preferred that the DNA gene construct be at a concentration of about 100 μg/ml or greater.

Selection of Transformants

The cells are placed on a recovery medium following electroporation and penetration of the gene construct into at least some of the transformation-competent cellls. The recovery medium is preferably a solid callus maintenance medium containing mannitol. The mannitol is preferably removed after one week and recovery on maintenance media is continued for an additional week before selection begins. The extent of the recovery period varies depending on the selection agent used, and/or the number of cells electroporated. The recovery period affords cells an opportunity to recover from electroporation and permits the cells to proliferate and stabilize so that adequate numbers of cells may be generated to facilitate selection and subsequent regeneration.

Identification and selection of those cells which both contain the gene construct and which are capable of regeneration to form plants is begun between l day and 4 weeks, preferably at about 1.5 - 2.5 weeks following recovery. There are two methods for selecting the transformed cells. The first comprises assaying transformed cells within calli or plants

regenerated therefrom for the expression of reporter genes or assessing the phenotypic effects of the gene construct, if any. The second, preferred method comprises identifying the transformed cells by growing the cells in the presence of an antibiotic, i.e., kanamycin, which is toxic for non-transformed cells, but allows growth of those cells transformed with and expressing the introduced gene construct including the selectable marker gene (i.e., neomycin resistance). Selection conditions are chosen to optimize growth and accumulation of the transformed cells while simultaneously inhibiting growth of the non-transformed cells. The selection agent's effect on cell viability, regeneration capacity and fertility is monitored and determined by experimentally establishing the concentration range which inhibits growth. This may be done by plotting a growth inhibition curve for the given selective agent and tissue being transformed.

A selection regimen may include sequential changes in the concentration of the agent and multiple selection cycles. Concentration and cycle length vary depending on the selection agent used. For instance, transformed suspension cultures may be selected by growing the cultures in the presence of kanamycin 200 mg/L for a period of 3-6 weeks followed by selection on 60 mg/l for 3 - 6 weeks.

Transformation of putative transformants is confirmed by phenotypic and/or genotypic analysis. For example, for collagen encoded by the gene construct of the invention, an immunologic specific assay using anti-collagen antibodies may be used. The presence of the gene construct may also be confirmed by conventional means, i.e., by Southern blot or by polymerase chain reaction (PCR). As the gene construct includes a plant expressible marker encoding the neomycin resistance gene, the plant cells are selected by culturing the plant cells in the present of neomycin.

Plant Regeneration and Seed Production

Transformed cell lines are regenerated into transgenic whole plants in a conventional manner well known in the art and the fertility of the resultant plants is determined. Transformed cell lines which test positive by genotypic and/or phenotypic analysis are placed on a media which promotes tissue differentiation and plant regeneration. Regeneration procedures typically include reducing the level of auxin and adding sucrose which discontinues proliferation of a callus and promotes somatic embryo development or other tissue differentiation. An example of a regeneration procedure is given in C. E. Green et al., Maize for Biological Research, Plant Molec. Biol. Assoc., Charlottesville, Va., pps. 367-372

(1982). The regenerated plants may be transferred to standard soil conditions and cultivated in a conventional manner. After the gene construct is stably incorporated into regenerated transgenic plants. it can be transferred to other plants by appropriate sexual crosses and selfs as described by M. Neuffer, ibid. at pages 19-30. Any of a number of standard breeding techniques may be used depending on the species to be crossed. The plants are grown and harvested using conventional procedures.

Analysis of R1 progeny

The initial plants generated from the transformed callus are termed the R0 generation or R0 plants. The term R1 progeny or the R1 generation refers to the seeds produced by various sexual crosses of the R0 generation plants. The progeny derived from the germination of R1 seeds are also referred to as the R1 generation.

Various tissues of the R1 generation are analyzed to confirm the successful transmission and inheritance of the gene construct in the sexual crosses described above. The analysis is performed as described above for analysis of the electroporated callus for evidence of transformation, except that plants and plant parts rather than callus are being analyzed.

Production of Commercial Hybrid Seed

It is advantageous to incorporate the gene construct of the invention into many varieties of hybrids which differ in maturity, yield, pest resistance, and other agronomic traits. Hybrids adapted to growth in one part of the corn belt may not be adapted to growth in other parts of the corn belt due to variations in such traits as maturity, disease, and insect resistance. It is therefore advantageous to integrate the gene construct into many parental hybrid lines to increase the hybrid combinations that may be produced containing the gene construct. This is accomplished by breeding programs in which backcrossing is performed by crossing the initial fertile, transgenic plant to an elite inbred line and then crossing the progeny back to the elite parent using conventional methods. Elite lines are pure breeding inbreds having commercially valuable traits as a result of classical breeding methods. Some of the progeny from this cross will carry the gene construct and some will not. The plants carrying the gene construct are then crossed again to the elite parent resulting in progeny which segregate once more. Repeated crosses are performed until the original elite line is converted to a genetically engineered line containing the gene construct and all other important traits present in the elite parent. A separate backcrossing program is employed for every elite line to be converted to a genetically engineered elite line. Using conventional breeding techniques, it is possible for

both parents of a hybrid seed corn to be homozygous for the gene construct. Corn breeding techniques required to transfer genes from one line or variety to another are well-known.

Alternatively, the gene construct may be delivered by electroporation into immature zygotic embryos preconditioned with a mild enzymatic treatment of immature embryos or into type I callus preconditioned with cutting and preplasmolysis of type I callus. D'Halluin et al., The Plant Cell 4:1495-1505 (1992). The next section describes an electroporation treatment of immature zygotic embryos, followed by a description of electroporation of type I callus, following the procedure of D'Halluin et al., supra.

Electroporation, Selection and Regeneration of Immature Zygotic Embryos

Freshly excised immature embryos of H99 or Pa91 are treated for 1-3 minutes with an enzyme solution containing CPW salts (Frearson et al, Dev. Bio. 33, 130-137 (1973)) supplemented with 10% mannitol, 5 mM Mes, pH 5.6 and 0.3% macerozyme (Kinki Yakult, Nishinomiya, Japan) or other combinations of pectin degrading enzymes. The embryos are then rinsed in appropriate buffers to remove residual enzymes. The transformation-competent cells are transferred to a microcuvette containing 200μL maize EPM electroporation buffer (80 mM KCl, 5 mM $CaCl_2$, 10 mM Hepes and 0.425 M mannitol, pH 7.2) and 10- 20 μg/ml gene construct and incubated for 1 hour. The cuvettes are then incubated in an ice bath for 10 min. and then transferred to an electroporation chamber. Several pulse protocols may be tested to determine the optimal conditions. For instance, electroporation may be carried out by discharging one pulse with a field strength of 375 V/cm from a 900 μF capacitor as described by D'Halluin et al., The Plant Cell 4:14951505 (1992). The pulse strength, capacitance, and electroporation apparatus are as described by Dekeyeser et al., Plant Cell 2:591-602 (1990). After electroporation, the embryos are transferred to recovery media (F medium or F medium supplemented with 0.2M mannitol) or directly to selection media (F medium supplemented with 0.2M mannitol and 200 μg/ml kanamycin) and cultured in the dark at 23 °C. Those transferred to recovery media are cultured 10 days then transferred to selection media. They are maintained on selection media with mannitol for 14 days then transferred to selection media without mannitol, but still containing kanamycin 200 μg/ml. The cells are then subcultured in 3 week intervals for 6-8 weeks. For regeneration, the developing embryogenic tissue is isolated and transferred to MS medium (Murashige et al., Physiol. Plant 15:473-497 (1962)) supplemented with 5 mg/L 6-benzyl-aminopurine for line H99 and 5 mg/L zeatin for line Pa91 and cultured at 23°C for 10 to 14

days. The embryogenic tissue is then transferred to MS medium without hormones and 6% sucrose. Developing shoots are transferred to half-strength MS medium with 1.5% sucrose for further development into plantlets. The plantlets are transferred to soil and grown to maturity in the greenhouse.

Electroporation, Selection and Regeneration of Type I Callus

Developing type I callus of Pa91 is cultured for about 2 months on Mah1VII substrate (N6 medium (Chu et al., Sci. Sin. Peking 18, 659-668 (1975)), supplemented with 100 mg/L casein hydrolysate, 6 mM l-proline, 0.5 g/L 2-(N-morpholino)ethanesulfonic acid (Mes), 1 mg/L 2,4-D, and 2% sucrose solidified with 1.6 g/L Phytage (Sigma), and supplemented with 0.75 g/L $MgCl_2$, pH 5.8) subcultured every 14 to 20 days. Embryogenic tissue is dissected from the developing type I callus and preconditioned by cutting in about 1.5 mm thick pieces in EPM buffer without KCl. and preplasmolysis for about 3 hours in this buffer. The 150 mg of callus fragments are then transferred to cuvettes containing 200μL of EPM supplemented with 80 mM KCl. Electroporation, selection and regeneration protocols are as described above for immature zygotic embryos.

Confirmation of Transformation of Callus

To show that callus lines grown on kanamycin selection medium have acquired the collagen compound gene, DNA is isolated from pieces of each kanamycin resistant callus line and from unselected control callus. Dellaporta et al., Plant Mol. Biol. Rep. 1, 19-21 (1983). Southern blot is performed on restriction enzyme digested DNA according to standard protocols as disclosed by Maniatis. however using the radiolabelled bovine collagen as a probe. Polymerase chain reaction is performed on DNA using primers to amplify selected sections of the bovine collagen gene. Standard protocols are employed as disclosed by the Perkin Elmer PCR manual. This confirms integration of the bovine collagen compound gene into the maize genome.

Immunological assays or in situ hybridizations are performed on tissue from plants showing integration. Frozen sections are prepared and stained with antibodies directed against type I collagen.

Extraction of the Collagen Compound

Progeny containing the desired collagen compound is identified by electrophoresis or Elisa technology. After cultivation, the transgenic plant is harvested to recover the produced

collagen compound or gelatin using conventional methods. This harvesting step may consist of harvesting the entire plant, or only part of the plant commercially harvested.

There are two major milling processes for corn. Dry milling of corn separates the germ from the endosperm. The endosperm is recovered in the form of coarse grits, corn meal and corn flakes, or it may be passed through fine rollers and reduced to corn flour.

The collagen compound may be extracted from the corn as collagen or gelatin by means of an existing corn wet-milling process. In the wet-milling process, after the corn kernels are cleaned to remove coarse material such as dust, chaff, cobs, stones and insects, the corn is steeped in large tanks of warm water generally containing acid and sulfur dioxide (a sulfurous acid solution) to soften the corn and render the starch granules separable from the protein matrix that envelopes them. If the temperature is sufficiently high, the collagen compound will be converted to gelatin during this step and will be extracted as gelatin by separation in the steep water or upon completion of the remaining steps described below. About 7% of the kernel's dry substance is leached out during this step, forming protein-rich steep water used as a feed ingredient. The softened kernels are coarsely ground to release the germs. The course grind produces a pulpy material containing germ, fiber (hull), starch, protein and collagen compound which is passed through a hydrocyclone separator where the germ is recovered. Because of their high oil content, the germs are lighter than the starch, protein, and fiber fractions and can easily be separated. The hulls and endosperm, the heavier particles, are discharged from the bottom of the hydrocyclone tube for further processing and the lighter germs are drawn off the top of the vortex. The germs are washed free of remaining starch, dried and the corn oil is removed by expelling or solvent extraction. The spent germs are used as a feed ingredient.

The slurry now contains the fiber (hulls) and the protein, starch and collagen compound fractions of the endosperm. The starch-protein-fiber-collagen compound slurry is subjected to an intense milling to release additional starch from the fiber. The fiber is then wet-screened from the starch-protein slurry, washed free of starch, and dried to form the major component of corn gluten feed. The best fiber can be additionally purified to become corn bran. The starch-protein-collagen compound slurry is separated into its component parts by passing it through combinations of high speed centrifuges and hydrocyclones to separate the heavier starch and collagen compound from the lighter protein. The protein fraction is filtered and dried in rotary or flash driers to yield corn gluten, which is rich in the corn

protein known as zein. The starch slurry is dewatered and dried to produced corn starch, which may be used as such or further converted into corn syrup by the hydrolytic action of acid or starch-splitting enzymes. The collagen compound fraction is separated by high speed centrifuge, dried and used in cosmetic, medical, reconstructive, therapeutic and industrial applications. Alternatively, the collagen compound may be recovered as a gelatin-corn starch combination in the same fraction using high speed centrifugation.

The presence of residual collagen in the corn plant after collagen/gelatin extraction does not affect its use in animal feed. In addition to being biocompatible, collagen is an ubiquitous protein already naturally present in animals.

While a number of embodiments of the present invention have been shown and described, it will be obvious to one skilled in the art that many changes and modifications may be made thereto without departing from the spirit and scope of the invention. It will be appreciated that the specific gene construct is not critical to the invention, and various other gene constructs that are the most suitable to particular hosts may be used.

1. A method of producing transgenic plants capable of expressing a collagen compound, said method comprising introducing a gene construct into cells of a plant, said gene construct comprising a DNA sequence coding for said collagen compound operably linked upstream (5') to a promoter sequence capable of initiating and directing transcription in plant cells of said DNA sequence, said gene construct integrated into the plant genome such that upon transformation said plant cells express said collagen compound,

regenerating transgenic plants from said plant cells; and

growing said transgenic plants for a time and under conditions sufficient to permit expression of said collagen compound.

2. The method of claim 1 further comprising the step of selecting said plant cells which have been transformed after the step of introducing said gene construct into cells of a plant and wherein transgenic plants are regenerated from said selected plant cells.

3. The method of claim 1 further comprising the step of recovering said collagen compound from said transgenic plants in the form of collagen.

4. The method of claim 1 further comprising the step of recovering said collagen compound from said transgenic plants as a gelatin-corn starch combination.

5. The method of claim 1 further comprising the step of recovering said collagen compound from said transgenic plants in the form of gelatin.

6. The method of claim 5 wherein said gelatin is extracted by a wet milling process.

7. The method of claim 1 wherein said transgenic plant is selected from the group consisting of rice, wheat, barley, oats. millet, sorghum amaranth, onion, asparagus and sugar cane, alfalfa, soybean, cotton, clover, sugarbeet, sunflower, carrot, celery, cabbage, cabbage, broccoli, brussel sprouts, radish, rapeseed, cucumber, pepper, canola, bean, lettuce, cauliflower, spinach, artichoke, pea, okra, squash, kale, collard greens, potato, tobacco, tomato. tea, coffee, guar, carob, Irish moss, giant kelp, and rockweed.

8. The method of claim 1 wherein said promoter sequence further comprises at one enhancer sequence to further stimulate transcription and expression.

9. The method of claim 1 wherein said gene construct further comprises one or more of:

a transcription termination sequence;

the maize alcohol dehydrogenase 1 intron 1 between said promoter sequence and said DNA sequence coding for said collagen compound; and

the rice actin 1 intron 1 sequences between said promoter sequence and said DNA sequence coding for said collagen compound.

10. The method of claim 1 wherein said transcription termination sequence comprises a polyadenylation signal.

11. The method of claim 10 wherein said polyadenylation signal is from a source selected from the group consisting of the cauliflower mosaic virus, nopaline synthase and octopine synthase genes.

12. The method of claim 1 wherein said gene construct further comprises a plant-expressible marker selected from the group consisting of selectable marker genes and reporter genes.

13. The method of claim 12 wherein said selectable marker encodes the neomycin resistance gene.

14. The method of claim 12 wherein said plant cells are selected by culturing said plant cells in the presence of a selective agent to which said selectable marker confers resistance.

15. The method of claim 14 wherein said selective agent is neomycin.

16. The method of claim 1 wherein said gene construct further comprises in the 5' to 3' direction of transcription, a second promoter sequence, a neomycin resistance gene, and a polyadenylation signal.

17. The method of claim 16 wherein said polyadenylation signal is from a source selected from the group consisting of the cauliflower mosaic virus, nopaline synthase and octopine synthase genes.

18. The method of claim 1 wherein said collagen compound is selected from the group consisting of procollagen, collagen and fragments thereof.

19. The method of claim 18 wherein said collagen compound is selected from the group consisting of bovine procollagen, bovine collagen I and fragments thereof, bovine collagen II through XIX and fragments thereof, human procollagen, human collagen and fragments thereof.

20. The method of claim 19 wherein bovine collagen I is selected from the group consisting of bovine collagen I alpha 1, bovine collagen I alpha 2 and fragments thereof.

21. The method of claim 1 wherein said promoter sequence comprises the 35S constituent of cauliflower mosaic virus.

22. A transgenic plant which produces a collagen compound produced by the method of claim 1.

23. A collagen compound-producing plant cell produced by the method of claim 1.

24. A collagen compound-producing plant cell descended from a plant cell produced by the method of claim 1.

25. Seed obtained by growing a transgenic plant which produces a collagen compound produced by the method of claim 1.

26. Collagen produced from plants or seeds.

27. Gelatin produced from plants or seeds.

28. A food product which includes gelatin produced from plants.

29. A gene construct capable of transforming a plant for producing a collagen compound in plant cells, said gene construct comprising a DNA sequence coding for said collagen compound operably linked upstream (5') to a promoter sequence capable of initiating and directing transcription in plant cells of said DNA sequence, said gene construct integrated into the plant genome such that upon transformation said plant cells express said collagen compound.

30. The gene construct of claim 29 which further comprises one or more of:

a transcription termination sequence;

the maize alcohol dehydrogenase 1 gene intron 1 between the promoter sequence and the DNA sequence coding for said collagen compound; and

the rice actin 1 gene intron 1 sequences between the promoter sequence and the DNA sequence coding for said collagen compound.

31. The gene construct of claim 29 wherein said transcription termination sequence comprises a polyadenylation signal.

32. The gene construct of claim 29 wherein said polyadenylation signal is from a source selected from the group consisting of the cauliflower mosaic virus, nopaline synthase and octopine synthase genes.

33. The gene construct of claim 29 wherein said promoter sequence further comprises at least one enhancer sequence to further stimulate transcription and expression.

21. The method of claim 1 wherein said promoter sequence comprises the 35S constituent of cauliflower mosaic virus.

22. A transgenic plant which produces a collagen compound produced by the method of claim 1.

23. A collagen compound-producing plant cell produced by the method of claim 1.

24. A collagen compound-producing plant cell descended from a plant cell produced by the method of claim 1.

25. Seed obtained by growing a transgenic plant which produces a collagen compound produced by the method of claim 1.

26. Collagen produced from plants or seeds.

27. Gelatin produced from plants or seeds.

28. A food product which includes gelatin produced from plants.

29. A gene construct capable of transforming a plant for producing a collagen compound in plant cells, said gene construct comprising a DNA sequence coding for said collagen compound operably linked upstream (5') to a promoter sequence capable of initiating and directing transcription in plant cells of said DNA sequence, said gene construct integrated into the plant genome such that upon transformation said plant cells express said collagen compound.

30. The gene construct of claim 29 which further comprises one or more of:

a transcription termination sequence;

the maize alcohol dehydrogenase 1 gene intron 1 between the promoter sequence and the DNA sequence coding for said collagen compound; and

the rice actin 1 gene intron 1 sequences between the promoter sequence and the DNA sequence coding for said collagen compound.

31. The gene construct of claim 29 wherein said transcription termination sequence comprises a polyadenylation signal.

32. The gene construct of claim 29 wherein said polyadenylation signal is from a source selected from the group consisting of the cauliflower mosaic virus, nopaline synthase and octopine synthase genes.

33. The gene construct of claim 29 wherein said promoter sequence further comprises at least one enhancer sequence to further stimulate transcription and expression.

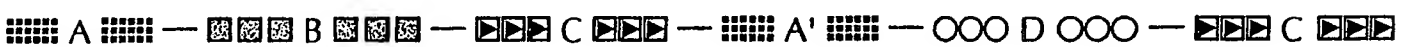
34. The gene construct of claim 29 which further comprises a plant-expressible marker selected from the group consisting of selectable marker genes and reporter genes.

35. The gene construct of claim 29 wherein said collagen compound is selected from the group consisting of bovine procollagen, bovine collagen I and fragments thereof, bovine collagen II through XIX and fragments thereof, human procollagen, human collagen and fragments thereof.

36. A transformed plant cell comprising the gene construct of claim 29.

37. A transgenic plant comprising cells containing the gene construct of claim 29 integrated in the genome of said cells.

FIG. 1



A= CaMV35S DOUBLE ENHANCER PROMOTER
A'= CaMV35S PROMOTER
B= COLLAGEN COMPOUND GENE
C= NOPALINE SYNTHASE TERMINATION/POLY A SIGNAL OR OCTOPINE SYNTHASE TERMINATION/POLY A SIGNAL OR CaMV35S TERMINATION/POLY A SIGNAL
D= NEOMYCIN RESISTANCE GENE

FIG. 2

A= CaMV35S DOUBLE ENHANCER PROMOTER
A'= CaMV35S PROMOTER
B= COLLAGEN COMPOUND GENE
C= NOPALINE SYNTHASE TERMINATION/POLY A SIGNAL OR OCTOPINE SYNTHASE TERMINATION/POLY A SIGNAL OR CaMV35S TERMINATION/POLY A SIGNAL
D= NEOMYCIN RESISTANCE GENE
E= RICE ACTIN 1 INTRON 1 OR CORN ALCOHOL DEHYDROGENASE INTRON 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/12049

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CABA, CAPLUS
search terms: collagen, gene, plant, expression, COL1A1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,956,282 A (GOODMAN et al) 11 September 1990, column 1, lines 5-61, column 2, lines 23-68, column 3, lines 1-68, column 4, lines 1-68, column 5, lines 1-60.	1-25, 29-37
Y	BERNARD et al. Nucleotide Sequences of Complementary Deoxyribonucleic Acids for the Pro-alpha1 Chain of Human Type I Procollagen. Statistical Evaluation of Structures That Are Conserved during Evolution. Biochemistry. 1983, Vol. 22, pages 5213-5223, especially pages 5216-5220.	1-25, 29-37

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 16 SEPTEMBER 1996	Date of mailing of the international search report 25 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Thomas Haas Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/12049

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YAMAMOTO et al. Construction of a Recombinant Bacterial Plasmid Containing Pro-alpha1(I) Collagen DNA Sequences. The Journal of Biological Chemistry. 25 March 1980, Vol. 255, No. 6, pages 2612-2615, especially page 2612.	1-25, 29-37
Y	GOLDBERG et al. Cloning and expression of a collagen-analog-encoding synthetic gene in Escherichia coli. Gene. 1989, Vol. 80, pages 305-314, especially page 306.	1-25, 29-37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/12049

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-25 and 29-37

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inter. .onal application No.
PCT/US96/12049

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12P 21/02; C12N 5/10, 5/14, 15/00, 15/09, 15/29.15/12, 15/82; A01H 1/00, 5/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

800/205, DIG 14, DIG 15, DIG 16, DIG 17, DIG 18, DIG 24, DIG 40, DIG 41, DIG 42, DIG 43, DIG 44, DIG 57, DIG 58, DIG 59, DIG 60, DIG 63; 536/23.5, 24.1; 435/320.1, 240.4, 172.3

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

800/205, DIG 14, DIG 15, DIG 16, DIG 17, DIG 18, DIG 24, DIG 40, DIG 41, DIG 42, DIG 43, DIG 44, DIG 57, DIG 58, DIG 59, DIG 60, DIG 63; 536/23.5, 24.1; 435/320.1, 240.4,

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claim(s) 1-25 and 29-37, drawn to transgenic plants comprising a heterologous DNA construct comprising a gene encoding collagen, classified in Class 800, subclass 205, for example.
Group II, claim(s) 26-28, drawn to collagen and collagen products classified in Class 530, subclass 356, for example.

The inventions listed as Groups I-II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
The inventions are not liked by a single special technical feature because the claims are drawn to a multitude of collagen genes with a multitude of DNA sequences encoding a multitude of collagen compounds.
The inventions do not share a single special technical feature because each involves physiologically and biochemically divergent products and processes not required by the other.
The invention of Group I involves DNA and transformation and regeneration techniques not required by Group II. The invention of Group II involves food products and food science techniques not required by Group I.
Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.